AD______________

AWARD NUMBER: W81XWH-10-1-0683

TITLE: Optimization of Assays to Assess Dendritic cell Activation and/or Energy in Ebola Infection

PRINCIPAL INVESTIGATOR: Christopher F. Basler, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, NY 10029

REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
September 2011

2. REPORT TYPE
Annual

3. DATES COVERED
9 August 2010 – 8 August 2011

4. TITLE AND SUBTITLE

Optimization of Assays to Assess Dendritic cell Activation and/or Energy in Ebola Infection

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0683

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Christopher F. Basler, Ph.D.

Email: chris.basler@mssm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mount Sinai School of Medicine
New York, NY 10029

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The immune responses during lethal virus infection and the correlates of protective immunity in vaccinated macaques are not well understood. This study aims to develop assays that can predict protection with the various vaccine platforms designed to provide immunity to filovirus (Ebolavirus (EBOV) and Marburgvirus (MARV)) infection. A secondary aim is to better understand the aspects of virus on the immune response in animals that receive no intervention. As part of these efforts, we have in (Task 1) begun to profile the functional and phenotypic status of immune cells in Ebola virus (EBOV)-infected non-human primates and (Task 2) to develop assays that will assess virus-induced immune dysregulation and identify strategies to overcome virus-induced immune dysregulation. The latter work focuses on macrophages and dendritic cells, which are important targets of Ebola viruses in vivo and are thought to mediate dysregulated immunity during infection in vivo 1-4.

15. SUBJECT TERMS
Filovirus, Ebola virus, immunity, antigen presenting cell, lymphocyte

16. SECURITY CLASSIFICATION OF: a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
U	U	U	UU	9	USAMRMC
					19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

	Page
Introduction……………………………………………………………..…….	**4**
Body…………………………………………………………………………..	**4**
Key Research Accomplishments………………………………………..……..	**7**
Reportable Outcomes…………………………………………………………	**8**
Conclusion……………………………………………………………………	**8**
References…………………………………………………………………….	**8**
Appendices……………………………………………………………………	**9**

INTRODUCTION: The immune responses during lethal virus infection and the correlates of protective immunity in vaccinated macaques are not well understood. This study aims to develop assays that can predict protection with the various vaccine platforms designed to provide immunity to filovirus (*Ebolavirus* (EBOV) and *Marburgvirus* (MARV)) infection. A secondary aim is to better understand the aspects of virus on the immune response in animals that receive no intervention. As part of these efforts, we have in **(Task 1)** begun to profile the functional and phenotypic status of immune cells in Ebola virus (EBOV)-infected non-human primates and **(Task 2)** to develop assays that will assess virus-induced immune dysregulation and identify strategies to overcome virus-induced immune dysregulation. The latter work focuses on macrophages and dendritic cells, which are important targets of Ebola viruses in vivo and are thought to mediate dysregulated immunity during infection in vivo 1-4.

BODY:

Task 1. The role of the Basler lab in Task 1 is to analyze flow cytometric data and to draw conclusions based on the data. All live EBOV work was done by USAMRIID personnel. The Basler lab designed study protocols and analyzed data.

A. Assessment of lymphocyte numbers and phenotypes in ZEBOV-infected NHPs.

- EBOV-infected macaques were bled by USAMRIID investigators on days 1-8 post-infection, and the samples were characterized individually. Note that the volumes of blood which can be obtained from an individual animal depend upon its weight and are limited by animal use protocols. Therefore, not all animals are bled on all days.

- The results indicate that total numbers of white blood cells appear to be relatively constant until very late in infection, when numbers drop. The lymphocyte population appears to expand by approximately two-fold on days 2 and 3 post-infection and then contracts by at least two fold as compared to levels present at the time of infection. Moreover, flow cytometry analysis of ex-vivo cells stained for lymphocyte markers at day 3-day 8 also demonstrate a continuous drop in the percentage of lymphocytes from day 3 to day 8. This lymphocyte population contraction may represent a mobilization, but it is also consistent with the previously reported lymphocyte apoptosis. We cannot exclude the possibility however that those cells which become activated emigrate out of the bloodstream into infected/noninfected tissues.

- When CD4+ T cells (CD3+, CD4+ cells) were stained ex vivo for the activation markers CD69 or CD62L, relatively little variation in levels of these surface markers was seen, suggesting that a significant proportion of these lymphocytes were not "classically" activated (i.e. upregulation of CD69 and downregulation of CD62L). Similar results were obtained with the CD3+CD8+ cells (data not shown).

- We have successfully begun to characterize immune cells in EBOV-infected primates by quantifying cell types. In future experiments, additional sampling should be performed at early times post-infection especially before and at timepoints when lymphocytes expand, a possible sign of lymphocyte stimulation and expansion and from draining lymph nodes. Of note, these observations are in contrast to those made by Bradfute et al. in mice, where activation, as evidenced by upregulation of CD44 and CD127 and downregulation of CD62L, was seen late (day 7) post-infection with mouse-adapted ZEBOV.

B. Analysis of the phenotypes of antigen-presenting cells in ZEBOV-infected NHPs.

- Antigen presenting cells were examined ex vivo by flow cytometry for the surface markers CD11c and CD14 as well as HLA-DR and CD86. The DCs (CD11c+ CD14- cells) exhibit a different phenotype than monocytes/macrophages (CD14+ HLA-DR+ cells).

- Macrophages seem to show some increase, later in infection, in HLA-DR and CD86 levels, while DC CD86 and HLA-DR expression drops off precipitously by day 8. Class II (HLA-DR+) and costimulatory marker upregulation are thought to be required for proper T helper cell stimulation.

C. Development of to develop methods to specifically identify, by flow cytometry, EBOV-infected cells. The goal of this work is to be able to differentiate infected and non-infected immune cells and to determine the phenotypic and functional status of each population.

- We sought to detect EBOV-infected cells isolated from infected macaques using an anti-GP antibody.

- To determine the identity of the cells infected with EBOV, PBMCs were stained with lineage markers and an anti-GP antibody (9C11). The 9C11 antibody and its isotype control were previously directly conjugated to the same fluorochrome. Thus the control stain for the PBMCs included a lineage marker stain and the isotype control.

- Previous in vitro performed experiments showed that some but not all of GFP expressing PBMCs (large cells) after infection with EBOV-GFP

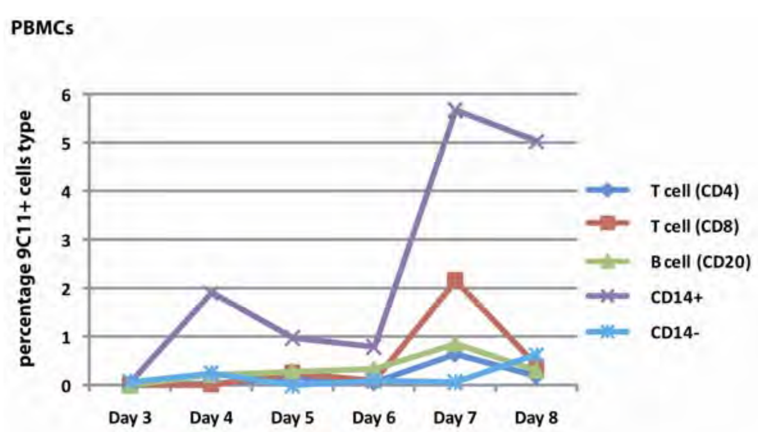

Figure 4. Percentages of the indicated cell types that stain positive for Zaire EBOV infection. PBMC from infected macaques were stained with ati-GP mouse monoclonal antibody 9C11 and with markers for the indicated cell types. The number of days post-infection for the samples are indicated.

(EBOV engineered to express GFP from infected cells) stained positive for 9C11. The percentage of cells deemed positive for cell surface EBOV GP was calculated as the percentage of 9C11 minus the percentage of cells positive for the isotype control. Isolated PBMCs from one to two infected nonhuman primates on days 3 to 8 were stained with lineage markers CD3, CD4, CD8, CD14 and either 9C11 or isotype control. The results are provided in the figure.

D. Profiling the functional and phenotypic status of immune cells in EBOV-infected NHPs. The following studies have been initiated and will continue in year 2.

- We have initiated a more detailed phenotypic analysis of the immune cells present in the spleens of infected non-human primates.

- We demonstrate steadily increasing numbers of EBOV-infected CD14+ cells in the spleens of infected animals.

Task 2. Develop assays that will assess virus-induced immune dysregulation and to identify strategies to overcome virus-induced immune dysregulation. Because EBOVs are reported to dysregulate host immune responses and because antigen presenting cells (dendritic cells, macrophages, monocytes) are important initiators of host immune responses, we sought to characterize, at BSL2, interactions between

A. EBOV entry into human antigen presenting cells.

- We developed assays that assess the ability of Ebola virus-like particles (EVLPs) to bind to human and mouse monocytes, macrophages and dendritic cells.

- We used the EVLPs to demonstrate that EBOV either cannot enter into human monocytes or can enter only at very low efficiencies.

- These data indicate that we have successfully developed assays to examine the earliest interactions, binding and entry, of Ebola viruses with immune cells. These data suggest that productive infection of monocytes should be very inefficient. Therefore, infected monocytes are unlikely to be important contributors to immune dysregulation in vivo.

B. EBOV entry into mouse antigen presenting cells. We alsostudied entry of EVLPs into murine peritoneal cells, because mouse-adapted Ebola viruses are lethal by this route but not by several other routes of infection. By identifying targets of virus entry and infection, we hope to gain insight into the connection between host cell tropism and virulence and to identify cells targeted by EVLPs to induce protective immune responses.

- We identified CD11b+ myeloid cells that include antigen-presenting cells (APC) as permissive for EBOV GP-mediated entry.

- Strikingly, however, we demonstrated that a previously demonstrated entry-defective mutant, GP-F88A, enters mouse, but not human APCs.

- Like wild-type (wt) GP, GP-F88A-mediated entry into mouse cells is cathepsin-dependent.

- GP-F88A entry not only occurs in murine peritoneal macrophages but also in macrophages or dendritic cells derived from mouse bone marrow monocytes.

- We have demonstrated that wild-type GP and GP-F88A-mediated entry into mouse macrophages and dendritic cells likely proceeds via macropinocytosis.

- We have demonstrated that GP-mediated entry into mouse macrophages and dendritic cells is abrogated by mutation of the fusion peptide, suggesting a requirement for the normal GP-mediated membrane fusion process.

- We have demonstrated that GP-mediated entry into mouse macrophages and dendritic cells occurs despite introduction of three mutations K114A, K115A, K140A, which were previously reported to impair GP binding to the (as yet unidentified) Ebola virus receptor. Thus suggests that interaction of GP with its receptor(s) on mouse macrophages and dendritic cells is different in mouse cells versus human cells.

- Because F88 occupies the same hydrophobic pocket as L111, I113, L122 and F225, we assessed the entry phenotype of mutants at residues 88, 111, 113, 122 and 225 into a variety of cell types.

- The mutants L111, I113, L122 displayed generally impaired entry and preferential entry into several cell types, but did not exhibit the strict tropism for mouse cells as did the F88A mutant.

- The mutant F225A displayed entry similar to wild-type GP.

- Cumulatively, the data from Task 2 demonstrate a role for GP residues F88, K114, K115 and K140 in EBOV host range restriction and suggest that EBOV may enter human and mouse cells by different mechanisms.

KEY RESEARCH ACCOMPLISHMENTS:

- We successfully characterized the phenotype and quantified lymphocytes during the course of EBOV infection in non-human primates.

- We successfully characterized the phenotype and quantified macrophages and dendritic cells during the course of EBOV infection in non-human primates.

- Our data support a model where immune cells are dysregulated during the course of EBOV infection in non-human primates and provide parameters that can be employed in the evaluation of vaccines and therapeutics.

- We developed and successfully employed assays to monitor attachment and entry of EBOV virus-like particles tagged with beta-lactamase.

- We demonstrated a strong preference for EBOV to enter macrophages and dendritic cells versus monocytes.

- We provided evidence that EBOV entry into mouse macrophages and dendritic cells canoccur by mechanisms distinct from entry into human macrophages and dendritic cells. These observations have implications for use of mice a model systems for filovirus pathogenesis and for evaluation of vaccines and antivirals.

REPORTABLE OUTCOMES:

Portions of Task 2 data were presented at the the 2010 Chemical Biological Defense Science and Technology Conference.

A manuscript describing our findings in Task 1 is in preparation.
A manuscript describing our findings in Task 2 has been written. We are now revising it in preparation for submission for peer review.

CONCLUSION:

The work completed thus far for Task 1 provides additional information regarding the status of the host immune system during the course of EBOV infection in unvaccinated and untreated non-human primates. The data provide a framework for the comparison of untreated versus treated and unvaccinated versus vaccinated animals. Therefore, this information can be used to help evaluate efficacy of vaccines and antivirals.

The work completed for Task 2 identifies specific targets of virus infection and suggests inhibition of virus entry as a potential means to mitigate the immune dysregulatory aspects of EBOV infection. It also notes differences in virus entry into human versus mouse antigen presenting cells which may influence the outcome of infection experiments.

REFERENCES:

[1] Geisbert, T.W. *et al.*, Apoptosis induced in vitro and in vivo during infection by Ebola and Marburg viruses. *Lab Invest* 80 (2), 171-186 (2000).

[2] Geisbert, T.W. & Hensley, L.E., Ebola virus: new insights into disease aetiopathology and possible therapeutic interventions. *Expert Rev Mol Med* 6 (20), 1-24 (2004).

[3] Bosio, C.M. *et al.*, Ebola and Marburg Viruses Replicate in Monocyte-Derived Dendritic Cells without Inducing the Production of Cytokines and Full Maturation. *J Infect Dis* 188 (11), 1630-1638 (2003).

[4] Mahanty, S. *et al.*, Cutting edge: impairment of dendritic cells and adaptive immunity by Ebola and Lassa viruses. *J Immunol* 170 (6), 2797-2801 (2003).

APPENDICES: None